



UNIVERSITI PUTRA MALAYSIA

**PURIFICATION AND CHARACTERISATION OF ENDOPEPTIDASE
PRODUCED BY LACTOCOCCUS LACTIS SUBSP. LACTIS RI 11**

WOO KWAN KIT

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**PURIFICATION AND CHARACTERISATION OF ENDOPEPTIDASE
PRODUCED BY *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* RI 11**

By

WOO KWAN KIT

**Thesis Submitted in Fulfilment of the Requirement for the
Degree of Master of Science in the Faculty of Food Science and Biotechnology
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July 2001



Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Master Science

**PURIFICATION AND CHARACTERISATION OF ENDOPEPTIDASE
PRODUCED BY *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* RI 11**

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July 2001

Chairman : Professor Gulam Rusul Rahmat Ali, Ph.D.

Faculty : Food Science and Biotechnology

In the this study, 10 isolates of Lactic Acid Bacteria (LAB) were isolated from *ikan rebus* (steam fish) purchased from a local market, was characterised by phenotypical and biochemical characteristics. Eight isolates were identified as *Lactococcus lactis* subsp. *lactis* and were evaluated for endopeptidase activity. As the endopeptidase activity of the crude cell extracts varied among isolates, only *Lc. lactis* subsp. *lactis* RI 11 was selected for further study. The optimum endopeptidase activity was at pH 7.5 and 45°C. The crude enzyme preparation was purified to apparent homogeneity by ammonium sulphate fractionation, anion and cation exchange chromatography and gel filtration chromatography. The

purification procedure has resulted 1.55 % yield and 2.36 purification fold. As the purified endopeptidase has 3 optimum temperatures (10°C, 50°C and 90°C) and pH (3.5, 6.5 and 9.5), it was likely that the endopeptidase consist more than one isoenzymes. The molecular mass of purified endopeptidase was approximately 14.15 kDa estimated with Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis. However, a lower molecular mass of 3.9 kDa was obtained from gel filtration chromatography. In terms of substrate specificity, the purified endopeptidase showed higher substrate affinity towards bradykinin with a K_m value of 0.029 mM, whilst, oxidised insulin B chain demonstrated the highest production rate with the V_{max} value of 10.52.

Abstrak ini dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**PENULINAN AND PENCIRIAN ENDOPEPTIDASE DIHASILKAN
DARIPADA *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* RI 11**

Oleh

WOO KWAN KIT

Julai 2001

Pengerusi : Profesor Gulam Rusul Rahmat Ali, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

Dalam penyelidikan ini, 10 pencilan Bakteria Asid Laktik (BAL) dipencilkan daripada ikan rebus yang dibeli daripada pasar tempatan, dicirikan melalui kaedah finotipikal dan biokimia. 8 pencilan dikenali sebagai *Lactococcus lactis* subsp. *lactis* dan diuji aktiviti endopeptidase. Disebabkan aktiviti endopeptidase dari ekstrak kasar berbeza antara pencilan, hanya *Lc. lactis* subsp. *lactis* RI 11 dipilih untuk menjalani kajian selanjutnya. Aktiviti optimumnya berada di pH 7.5 dan suhu 45°C. Endopeptidase kasar yang disediakan kemudian ditulinkan sehingga mencapai tahap homogenisasi melalui kaedah pemendakan amonium sulfat, kromatografi penukar anion dan kation dan kromatografi penurasan gel. Kaedah

penulinan ini telah menyebabkan 1.55 % hasil dan 2.36 tahap penulinan. Disebabkan activity endopeptidase tulin menunjukkan tiga suhu optima (10°C, 50°C dan 90°C) and pH (3.5, 6.5 dan 9.5), ia dipercayai mengandungi lebih daripada satu isoenzim. Ketumpatan molekul dianggarkan sebanyak 14.15 kDa melalui kaedah analisa gel elektroforesis sodium dodesil sulfat, manakala ketumpatan molekul yang lebih rendah, iaitu 3.9 kDa, dianggarkan dengan kromatografi penurasan gel. Dari segi kespesifikan substrat, bradikinin menunjukkan afiniti substrat yang tinggi dengan nilai K_m 0.029 mM, sebaliknya, B-insulin teroksida menunjukkan kadar penghasilan yang paling tinggi dengan nilai V_{max} sebanyak 10.52.

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To my beloved family, I dedicated this thesis-for without your love and support I will not be here today to pursue my dreams.

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I certify that an Examination Committee met on 25th July 2001 to conduct the final examination of Woo Kwan Kit on her Master of Science thesis entitle “Purification and Characterisation of Endopeptidase Produced by *Lactococcus lactis* subsp. *lactis* RI 11” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows :

Suraini Ph.D.
Faculty of Food Science and Biotechnology
University Putra Malaysia
(Chairman)

Gulam Rusul Rahmat Ali, Ph.D.
Professor,
Faculty of Food Science and Biotechnology
University Putra Malaysia
(Member)

Foo Hooi Ling, Ph.D.
Faculty of Food Science and Biotechnology
University Putra Malaysia
(Member)

Son Radu, Ph.D.
Associate Professor
Faculty of Food Science and Biotechnology
University Putra Malaysia
(Member)



MOHD. GHAZALI MOHAYIDIN, Ph.D.
Professor/Deputy Dean of Graduate School,
Universiti Putra Malaysia

Date : 30 AUG 2001.



This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.



AINI IDERIS, Ph.D.
Professor
Dean of Graduate School,
Universiti Putra Malaysia

Date : 08 NOV 2001

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



WOO KWAN KIT

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LIST OF ABBREVIATIONS

Ala	Alanine
α -casein	Alpha casein
α_{s1} -CN(f1-23)	Alpha casein fragment 23
PepA	Aminopeptidase A
PepC	Aminopeptidase C
$(\text{NH}_4)_2\text{SO}_4$	Ammonium sulphate
ANOVA	Analysis of variance
β -casein	Beta casein
β -naphthylamides	Beta-naphthylamides
CFU/g	colony forming unit
Co^{2+}	Cobalt ion
Cu^{2+}	Cupric ion
$^{\circ}\text{C}$	Degree celcius
DNA	Deoxyribonucleic acid
PepO	Endopeptidase O
FP	Energy rich phosphate bond
FPLC	Fast protein liquid chromatograph
<i>g</i>	G-force
Glu	Glutamine
His	Histidine
HCl	Hydrochloride
H_2O_2	Hydrogen peroxide
κ -casein	Kappa casein
kDa	Kilo dalton
K_m	Mechaelis-Menten constant
LAB	Lactic acid bacteria
<i>Lb.</i>	<i>Lactobacillus</i>
<i>Lc.</i>	<i>Lactococcus</i>
Lys	Lysine
Mn^{2+}	Manganase ion
MPa	Mega Pasca
MEP	Metalloendopeptidase
μm	Micrometer
mg/ml	Miligram per mililitre
min.	Minute
ml	Mililitre
μl	Microlitre
μM	Micromolar
mM	Milimolar
<i>Mr</i>	Molecular Mass
M	Molar
MRS	deMan Rogosa Sharpe
NOP	Neutral Endopeptidase
N	Normal

PepX	Peptidase X
Pep N	Peptidase N
%	Percent
% TA	Percentage of titrable acid
Phe	Phenylalanine
PAGE	Polyacrylamide gel electrophoresis
Pro	Proline
PMF	Protein motive force
Re Q	Resource Q
Re S	Resource S
rRNA	Ribosomal Ribonucleic acid
rpm	Rotation per minute
NaCl	Sodium chloride
NaH ₂ PO ₄	Sodium dihydrogen phosphate
SDS	Sodium dodecyl sulphate
NaOH	Sodium hydroxide
subsp.	Subspecies
TCA	Trichloroacetic acid
TPC	Total plate count
XPdap	x-prolyl dipeptidylaminopeptidase
EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethylsulfonyl fluorida
V	Volt
v/v	volume per volume
V _{max}	Maximum reaction velocity
w/v	weight per volume

CHAPTER I

GENERAL INTRODUCTION

Lactic acid bacteria (LAB) are well known as starter cultures and are used in many fermented products. LAB possess a proteolytic system, which is closely related to the flavour of fermented foods. The amino acids produced during proteolysis contribute to the flavour of fermentation foods (Ohhira *et al.*, 1990).

LAB is Gram positive bacterium, multiple-amino acid auxotroph and requires essential amino acids for growth (Chen and Steele, 1998). Hence, LAB possesses an active proteolytic system to facilitate specific nutritional requirements from exogenous amino acids (Reiter and Oram, 1962). There are major differences in proteolytic ability among different species of LAB. There are many species of LAB that are known to possess proteolytic systems that allow them to grow on protein rich substrates such as meat, plants and milk.

The proteolytic system of LAB is a complete system and has been extensively studied for several years (Monnet, 1995; Tan *et al.*, 1993; Kok, 1991). A cell envelope associated proteinase, which is essential for the optimal growth of bacteria

in milk, is the first enzyme to degrade casein. The proteinase hydrolyses proteins into peptides, which provide suitable chain length, will be transported through the plasma membrane using an oligopeptide transport system. The initial cleavage of large casein fragment to smaller peptides by endopeptidase and subsequent degradation by several aminopeptidases is a fundamental route for casein utilisation (Atlan *et al.*, 1989; Niven, 1991). For *Lactococcus lactis*, a dozen different peptidases have been described and characterised. All of them seem to have an intracellular location and potentially play a role in the nutrition of the cell (Monnet, 1995).

Endopeptidases are a group of enzymes that act on smaller polypeptides or oligopeptides, they are not proteinases because they do not hydrolyse peptide bonds in protein. They are usually call oligopeptidases and constitute as a subgroup of the endopeptidase family (Barret and Rawlings, 1992).

Endopeptidase activity also contributes to the production of bioactive peptides. These bioactive peptides perform both biological and physiological functions. According to Kim *et al.* (1995), peptides derived from cheese slurry prepared by *Lc. lactis* subsp. *lactis* as starter culture have anti-carcinogenic properties.

In mammals, oligopeptidases are involved in the later part of the degradation of proteins, by hydrolysing peptides in the cytoplasm and also have a regulatory function on bioactive peptides such as bradykinin, enkephalin precursors, lutein, neurotensin and angiotensin. In *Salmonella typhimurium*, which is the Opd A play a role in protein turn over and hydrolyses the intermediates formed during protein breakdown has been studied by Vimr *et al.* (1983). In *Escherichia coli*, Opd A is associated with the membrane-bound proteinase IV (Novak *et al.*, 1986).

There are two characteristics that differentiate these lactic acid bacteria from many other proteolytic microorganisms. Firstly, LAB are fastidious organisms with multiple amino acid requirements and as a consequence, their growth is critically dependent on efficient systems for the degradation of proteins and the transport of amino acids and small peptides. Secondly, several LAB contain proteolytic system that is highly specific and results in the production of unique peptides (Kok and De Vos, 1994).

Endopeptidases of LAB have been less extensively studied than exopeptidases; however some oligopeptidases have been purified. Two different endopeptidases (designated LEP I and II), showing different substrate specificities on a range of oligopeptidases (Yan *et al.*, 1987a, b), have been reported in *Lactococcus lactis* subsp. *cremoris* H61. Endopeptidases, subsequently designated as Pep O (Mierau *et al.*, 1993) and Pep F (Monnet *et al.*, 1994), have been purified from *Lactococcus lactis* subsp. *cremoris* Wg2 (Tan *et al.*, 1991) and genetically characterised. This